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OPIOID ADDICTION AND PREGNANCY: POTENTIAL EFFECTS OF SUBSTITUTION THERAPIES ON DEVELOPMENTAL MYELINATION

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OPIOID ADDICTION AND PREGNANCY: POTENTIAL EFFECTS OF
SUBSTITUTION THERAPIES ON DEVELOPMENTAL MYELINATION

A Dissertation submitted in partial fulfillment of the requirements for the degree of
Master of Science at Virginia Commonwealth University.

by

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LIST OF ABBREVIATIONS

cAMP	3'-5'-cyclic adenosine monophosphate
CDM	Chemically defined medium
CNPase	2',3'-Cyclic nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CREB	Cyclic AMP response element-binding protein
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH ₂
DAG	Diacylglycerol
DMEM/F-12	Dulbecco's modified Eagle's medium/ Ham's F-12 medium
ERK	Extracellular-signal-regulated kinase
GABA	Gamma-aminobutyric acid
GDP	Guanosine diphosphate
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
IP ₃	Inositol trisphosphate
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
MBP	Myelin basic protein
MOG	Myelin oligodendrocyte glycoprotein
NMDA	<i>N</i> -methyl <i>D</i> -aspartate ionotropic glutamate receptor
ORL-1	Opioid receptor-like 1 receptor
PBS	Phosphate buffered saline
PLP	Proteolipid protein

PNS	Peripheral nervous system
SDS	Sodium dodecyl sulfate
T ₃	Thyroid hormone
TCA	Trichloroacetic acid

ABSTRACT

OPIOID ADDICTION AND PREGNANCY: POTENTIAL EFFECTS OF SUBSTITUTION THERAPIES ON DEVELOPMENTAL MYELINATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of
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While most cells of the central nervous system (CNS) express opioid receptors, the role of the endogenous opioid system in CNS development remains poorly understood.

Identification of opioid functions during brain maturation is particularly crucial in light of the increasing trend in opioid abuse and the use of opioid drugs during pregnancy. New substitution therapies in pregnant opioid addicts include buprenorphine, a mu opioid receptor partial agonist and kappa opioid receptor antagonist. However, while clinical studies demonstrated buprenorphine efficacy

in reducing neonatal withdrawal symptoms, there is a lack of information on the potential effects of this drug on brain development.

Previous work from our laboratory has shown that perinatal exposure to buprenorphine induces dose-dependent alterations in rat brain myelination. These time-specific effects suggested that both therapeutic and supra-therapeutic doses of the drug could alter the normal pattern of oligodendrocyte development. In support of this hypothesis, this thesis work has now found that buprenorphine exerts direct actions on the oligodendrocytes that are highly dependent on both the drug dose and stage of cell differentiation. When exposed to buprenorphine, oligodendrocyte progenitors isolated from 3-day-old rat brain exhibit increased cell proliferation. In contrast, treatment of more mature oligodendrocytes obtained from 9-day-old rat brain induces dramatic dose-specific effects on cell process network extension and membrane outgrowth. These later effects are accompanied by significant parallel changes in the expression of the four major splicing isoforms of myelin basic protein (MBP), a critical component of the myelin membrane and mature myelinating oligodendrocytes. Furthermore, similar dose-specific effects on MBP expression are also elicited by methadone, a mu opioid receptor agonist already approved for the treatment of pregnant opioid addicts. Experiments with CTOP, a highly selective antagonist of the mu opioid receptor, further contribute to the idea that this receptor subtype plays an important function in controlling oligodendrocyte maturation.

These findings underscore the potential effects of opioid exposure during brain maturation and further indicate an important regulatory role of the endogenous opioid system in the control of oligodendrocyte development and myelination.

INTRODUCTION

Epidemiological data show an increasing incidence of opioid abuse (Compton and Volkow, 2006), a critical problem among pregnant addicts. Surprisingly, little is known about the effects of these drugs on child development, although several clinical studies suggest that their use during pregnancy may interfere with the formation of the central nervous system (CNS). Infants exposed to opioids *in utero* exhibit reduced head circumference, decreased attention, altered fine motor coordination, and a greater risk of sudden infant death syndrome (Rosen and Johnson, 1982, Marcus et al., 1984, Kandall et al., 1993). In addition, longitudinal studies found that these children showed heightened activity, impulsivity, and reduced attention span, suggesting underlying neurological problems (Hutchings, 1982). However, the effects of perinatal exposure to opioids on brain development are difficult to assess because behavioral and cognitive outcomes in children are known to be highly influenced by environmental, educational, and lifestyle factors (Ornoy et al., 2001).

The current FDA approved standard treatment for opioid addiction during pregnancy is substitution therapy with methadone, even though this mu opioid receptor agonist has been linked to neonatal abstinence syndrome upon discontinuation (Fischer et al., 2006, Lejeune et al., 2006). One alternative possibility is the use of buprenorphine. This mu partial agonist and kappa antagonist has been approved to treat opioid-dependent adults in the United

States and is also being used experimentally in pregnant addicts in this and several other countries. Clinical trials indicate that buprenorphine is effective in the prevention of “street opioid” abuse by pregnant addicts as well as reducing the incidence and severity of neonatal abstinence syndrome (Jones et al., 2005, Ebner and Wiedmann, 2006). However, as indicated above, not much is known about the potential effects of these treatments on the developing CNS.

The brain is most vulnerable to insults during development when critical periods of network establishment and refinement are underway. Recent studies suggest that a major factor in the coordination of these events is the endogenous opioid system. Both neurons and glia express opioid receptors and endogenous opioid peptides in a developmentally controlled manner (Wu et al., 1997, Ikeda et al., 1998, Knapp et al., 1998, Leslie et al., 1998, Miller and Azmitia, 1999, Kivell et al., 2004). In this regard, perinatal exposure of rats to buprenorphine has been shown to delay the generation of cholinergic neurons (Robinson, 2002) and to reduce the expression of nerve growth factor in the striatum (Wu et al., 2001), underscoring the importance of further research on the actions of this drug in CNS development.

Interestingly, previous work from our laboratory has shown that buprenorphine affects the formation of myelin (Sanchez et al., 2008), the multilamellar membrane that insulates axons allowing the rapid saltatory conduction of nerve impulses. In the CNS, the myelin membrane is produced by oligodendrocytes, a type of glial cell, which is the center of these studies and will be described in detail in the following sections of this thesis. The synthesis of

myelin is under control of multiple growth factors and hormonal signals as well as cell-cell interactions, making this a particularly vulnerable process during CNS development. The observed effects of buprenorphine on myelination beg further investigation, as the roles of opioid receptors in oligodendrocyte development and *in vivo* myelination are unknown.

This work investigates the direct effects of opioids on developing oligodendrocytes *in vitro*. Characterizing these specific effects will help to further understand the *in vivo* role of the endogenous opioid system in controlling myelination as well as provide important clues for the development of therapeutic interventions for pregnant opioid addicts.

First, a background of opioids and glia will be given before we investigate their coordinated effects on oligodendrocyte development and function.

OPIOIDS

Opioids comprise a family of drugs that has long been used to relieve various types of pain. Opiates were originally defined as derivatives of the opium poppy (*papaver somniferum*). However, the modern term “opioids” encompasses opiates as well as synthetic and endogenous molecules, all of which are able to interact with opioid receptors. Today, these compounds are widely used for their analgesic properties as well as their inhibitory effects on the digestive system.

Opioids have been used medicinally for thousands of years. The use of opium for pain management and dysentery treatment existed in antiquity, but did not become widespread until the mid-19th century. The global opium trade originated in the Middle East and was facilitated by the imperial campaigns of the British Empire. Soon, opium use spread to the Far East as well as Western Europe. Opioids had previously been available in the United States, but at the turn of the century their use expanded dramatically. Heroin was widely used to relieve cough, as well as a range of maladies from digestive problems to menstrual pain. However, these substances are characterized by their high potential for abuse, resulting in serious clinical manifestations and making users vulnerable to severe withdrawal upon cessation. As they became readily available, the incidence of opiate abuse rose. By 1914, the US government acknowledged the growing problem and brought opioids under federal regulation by passing the Harrison Act (Acker, 2002). Presently, opioids are clinically available by prescription only. Yet opioid abuse remains a problem worldwide.

Physiological Effects of Opioids

Opioids are the most effective drugs known for pain relief. At therapeutic doses, they successfully suppress pain with a minimal effect on other sensations. Opioid analgesia is specific to ‘second pain’: the dull ache after noxious stimulus, or ‘first pain’ (Cooper et al., 1986). This may explain the evolutionary significance of the endogenous opioid system—dulling second pain does not hinder

perception of future noxious stimuli. Opioids have also been shown to reduce emotional pain, which is a major factor in their high potential for addiction.

These drugs cause marked physiological changes when consumed at analgesic doses. Decreased body temperature and pituitary function, respiratory depression, pupil constriction, suppressed cough reflex, nausea, decreased gastrointestinal secretions and motility, and suppression of the immune system are all observed. In neurons, opioids presynaptically inhibit the release of small nociceptors via the inhibition of calcium channels (Taddese et al., 1995).

Opioid Subtypes

Naturally Occurring Opiates

The naturally occurring opiates are alkaloids derived from the papaver somniferum plant. Morphine is the predominant type, but codeine and thebaine can also be derived from the plant (Martin, 1967).

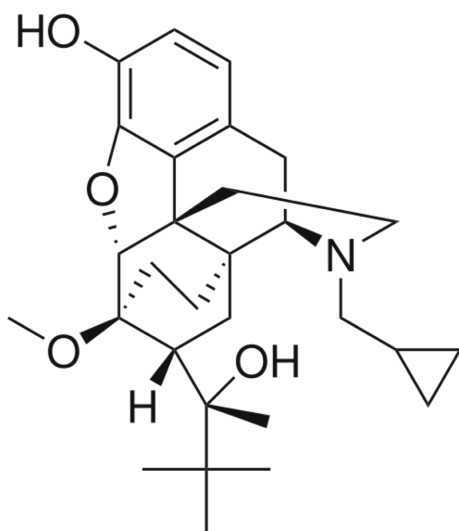
Synthetic Opioids

Synthetic opioids are the largest subtype and constitute the majority of molecules within this group that are used for pharmaceutical purposes (Portenoy, 1993). These substances have molecular structures analogous to those of naturally occurring opioid alkaloids. Examples include diacetylmorphine (heroin), hydrocodeine, oxycodone, fentanyl, methadone, and buprenorphine. Along with morphine, many synthetic derivatives are used clinically for pain management.

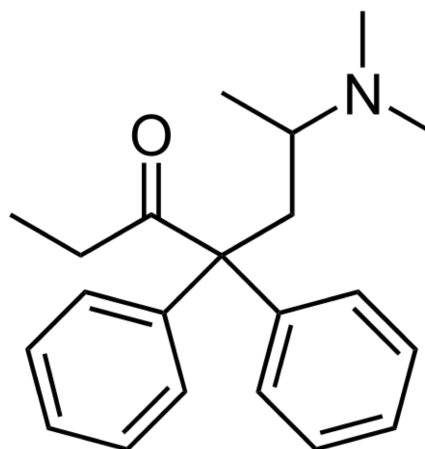
Buprenorphine and methadone, notably, are used to treat opioid addiction by mitigating withdrawal symptoms through replacement therapy (Figure 1). These two drugs will be described in more detail in following sections of this thesis as their administration to pregnant opioid addicts has the potential of affecting crucial steps along brain development.

Figure 1. The molecular structures of buprenorphine and methadone. These synthetic opioids are commonly used in replacement therapy.

Buprenorphine



Methadone



Endogenous Opioids

Interestingly, the human body produces endogenous opioid peptides that were discovered as a result of initial studies investigating exogenous opioid actions and the role of different opioid receptors. Many of the original studies used high-affinity alkaloids to identify opioid receptor types. Morphine was used to identify the mu receptor, ketocyclazocine for the kappa receptor (Lord et al., 1977). However, a stimulation-induced analgesia was observed in animals that could be reversed with the administration of naloxone, a known opioid receptor antagonist (Mayer et al., 1971). The first two peptide candidates for mediating this response were methionine- and leucine-enkephalin, endogenous peptides consisting of five amino acid residues (Waterfield et al., 1976). Shortly thereafter, β -endorphin and dynorphin were isolated. The enkephalins, endorphins, and dynorphins make up the three distinct groups of endogenous opioid peptides. The constituents of these three groups differentially bind the mu, delta, and kappa opioid receptors with varying affinity. The ORL-1 receptor only binds the endogenous peptides nociceptin and orphanin FQ (Meunier et al., 1995, Reinscheid et al., 1995).

Opioid Receptors

All opioids bind to at least one type of opioid receptor. This family of receptors is comprised of the mu opioid receptor, the kappa opioid receptor, the delta opioid receptor, and the opioid receptor-like 1 receptor (ORL-1). This

nomenclature was derived from radiolabeled ligand affinity studies. For example: the mu receptor was defined and characterized by its high affinity for morphine. These studies revealed stereospecific, saturable binding kinetics (Pert et al., 1973), along with the differential distribution of mu, delta, and kappa in the brain with a high concentration present in areas relevant to pain, including the periaqueductal grey, medial thalamus, and amygdala (Hiller et al., 1973, Kuhar et al., 1973, Pert et al., 1976). ORL-1 was discovered decades later through sequence homology studies. Like the other receptors, it was cloned and shown to match the endogenous receptor. Originally, it was named the N/OFQ receptor for its endogenous ligands: nociceptin and orphanin FQ (Butour et al., 1997). It differs from mu, delta, and kappa receptors by its negligible response to naloxone—a potent antagonist to the three other receptors. In addition, ORL-1 does not bind any of the other endogenous opioid peptides.

The mu, delta, and kappa opioid receptors exhibit both high structural and functional homology. These molecules are G-protein coupled receptors (GPCR) with seven transmembrane domains spanning the phospholipid bilayer. The receptor is coupled to a heterotrimeric G-protein that acts as the transducer between receptor and the eventual effector response. These G-proteins are composed of three subunits whose activity is regulated by allosteric interaction with guanosine triphosphate (GTP) and guanosine diphosphate (GDP). When bound by an agonist, opioid receptors inhibit adenylyl cyclase activity through activation of the G_i/G_o G-protein. These G-proteins can also affect phospholipase C, mitogen-activated protein kinase (MAPK), and ion channel activity. G_i/G_o

appears to be the G-protein most prominently involved in these primary transduction mechanisms, but opioid receptors have also been linked to other G-proteins that mediate secondary transduction mechanisms (e.g. G_q , G_{11} , G_{12} , G_{13} , and G_{16}) (Lee et al., 1998, Ho et al., 2002). These primary and secondary effects alter levels of second messengers like cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG), and inositol triphosphate (IP_3), signaling molecules which can regulate the expression and activation of various kinases and transcription factors and eventually cause changes in gene regulation. The mu, delta, and kappa receptors all have constitutive levels of activity, so responses to ligand binding are modulatory as opposed to binary.

GPCRs are subject to desensitization and endocytosis with chronic opioid use. β -arrestin is recruited to the receptor after it is phosphorylated by G protein receptor kinase and decouples the receptor from the G protein. Unlike other models of chronic receptor activation, opioid receptor levels do not decrease dramatically over time. One hypothesis is that the GPCR becomes dissociated from its downstream effectors and therefore is less effective in inhibiting adenylyl cyclase (Christie et al., 1987).

GLIAL CELLS

The neuronal doctrine has dictated the study of brain function and brought about our current understanding of the nervous system. This dogma proffers that neurons and the synapses adjoining their networks are the primary units of the

nervous system structure and function. In recent years, however, this view has been borne out as incomplete. Glial cells were characterized by Virchow as “nervenkitt” (nerve glue) upon their discovery, but they are increasingly implicated in signaling, development, plasticity, and other functional aspects of the brain. Recent evidence suggests that glial networks act in concert with neurons to refine and control the nervous system.

Glia Characteristics

Glia consist of five cell types: astroglia, microglia, oligodendrocytes, ependymal cells of the CNS, and the Schwann cells of the peripheral nervous system (PNS). Each of these cell groups possesses an array of functions which are integral to nervous system physiology. Along with neurons, glial types constitute the main categories of cells in the CNS. Neurons differ from glia in their ability to conduct action potentials. However, the inability of glia to propagate these all-or-none electrical signals does not designate them as static entities; rather, they refine circuits by limiting and directing electrical propagation.

Surprisingly, glia are the predominant cell type while neurons account for only 20%-40% of all cells in the CNS. The macroglia, of ectodermal origin, are comprised of astrocytes (60-80%), ependymal cells (5%), and oligodendrocytes (5%-10%). Microglia originate from the mesoderm and under normal non-inflammatory conditions they make up 10% of the CNS glia.

Glial morphology and distribution was thoroughly described in the 19th century by histologists Camillo Golgi, Santiago Ramón y Cajal, and Pio Del Rio Hortega. Yet, investigation of their functional importance was not conducted until recent decades. Glial coupling and networks were first observed in the 1960's (Kuffler and Nicholls, 1966, Brightman and Reese, 1969). However, technological improvements were necessary for advancing the study of glial function, and, in 1984, receptors for the neurotransmitters glutamate and gamma-aminobutyric acid (GABA) were discovered in astrocytes and oligodendrocytes (Bowman and Kimelberg, 1984, Kettenmann et al., 1984a, Kettenmann et al., 1984b). In recent years, glia have shed their label as the mortar of the CNS and proven to play a variety of functional roles. Astrocytes are known to express multiple receptors and enzymatic systems whose main functions are to maintain nervous system homeostasis and to limit neurotransmitter availability. These cells are also implicated in the induction and formation of the blood brain barrier and developmental synaptogenesis (Barker and Ullian, 2010, Giaume et al., 2010, Christopherson et al., 2005).

On the other hand, oligodendrocytes are responsible for making the myelin membrane in the CNS. Schwann cells represent their counterparts in the PNS. Oligodendrocytes are the center of these studies and therefore, they will be described in greater detail in the following sections.

Importantly, The ability of both astrocytes and oligodendrocytes to respond to neurotransmitters (Karadottir et al., 2005); together with the newly established presence of glial networks (Verkhratsky et al., 2009), has profoundly

implicated glia in current neurobiological studies, challenging many preconceived ideas on the organization and functioning of the CNS.

The Myelin Membrane

Myelin Structure

Myelin is a specialized multilamellar structure that ensheaths axons in the vertebrate nervous system. It is a three-dimensional structure formed by the extension and concentric wrapping of the oligodendroglial membrane, remaining continuous with the oligodendrocyte. A single oligodendrocyte can myelinate 10-30 axons simultaneously and multiple oligodendrocytes are responsible for generating the different myelin internodes along a single axon, resulting in a highly intricate glial-neuronal network (Baumann and Pham-Dinh, 2001).

A periodicity to myelin exists; it is generated along the axons as 150-200 nanometer-long longitudinal segments called internodes (Butt and Ransom, 1989). These internodes are flanked by the nodes of Ranvier: axonal areas lacking myelin but with an increased density of ion channels. As discussed later, this periodic distribution of ion channels is determined by the presence of internodes, and together with the insulating properties of myelin, is the basis for the rapid saltatory conduction of electrical signals—the impulse jumps from node to node.

Cross sections of myelinated axons visualized by electron microscopy (Figure 2) show that myelin appears as a sequence of dark and less dark lines separated by lighter zones. The dark line—known as the major period line—is formed by the fusion of inner cytosolic surfaces of the oligodendrocyte membrane. On the other hand, the lighter line is the intraperiod or minor dense line, formed by the compaction of the membrane's outer leaflets. Uncompacted areas in the myelin internode are believed to be essential for cellular transport and myelin maintenance. Because myelin is a dynamic structure its components must constantly be replaced to account for normal membrane turnover. These areas of cytoplasm are known as paranodal loops and contain mitochondria, free polysomes, and smooth endoplasmic reticulum for protein and lipid synthesis. Paranodal loops tend to be located near nodal regions and form transverse bands with the periaxonal membrane, which strengthen the connection between the axon and paranode (Ichimura and Ellisman, 1991). This is markedly different from myelin in the internodal region, which is separated from the periaxonal space by an extracellular gap.

The Role of Myelin in Nerve Transmission and Axonal Integrity

In unmyelinated nerve fibers, conduction velocity is mainly dependent on axonal caliber. In contrast, myelinated fibers allow for rapid conduction velocity and transmission fidelity without a corresponding increase in axonal caliber. This is achieved by a fundamental difference in conduction mechanism between both

types of fibers. In unmyelinated fibers, a uniform distribution of ion channels throughout the axolemma results in local circuits propagating depolarization in a continuous manner along the axon. By contrast, myelinated axons only expose the axonal membrane to the extracellular space at nodal regions. Depolarization cannot move continuously along the axon due to the high membrane resistance and absence of ion channels in myelinated internodes. Therefore, depolarization can only occur at nodes where ion channels are exposed. The impulse jumps from node to node. This is known as 'saltatory' conduction (Figure 3).

Furthermore, several lines of evidence indicate that myelin is also a key factor in axonal development. It has been shown that myelination can regulate axonal caliber in the optic nerve as well as neurofilament spacing and phosphorylation, both of which control axonal radial growth (Hsieh et al., 1994, Sanchez et al., 1996). Moreover, several lines of evidence have shown that myelination also exerts control over the distribution of the voltage-gated ion channels responsible for impulse conduction. Different studies have shown that the myelin membrane functions as a barrier that limits the lateral movement of ion channels in the axolemma, restricting the localization of voltage-gated sodium channels to the nodes of Ranvier while fast voltage-gated potassium channels are concentrated in the juxtaparanode (Rasband and Trimmer, 2001, Poliak and Peles, 2003, Rosenbluth, 2009).

In mature vertebrates, myelin also functions as an inhibitor of neurite outgrowth (Domeniconi et al., 2002). By dampening plasticity, myelin may act to refine late-developing tracts and preserve established myelinated regions.

Moreover, evidence from demyelinating diseases like multiple sclerosis suggest that both oligodendrocyte and myelin play an important role in axonal protection and survival (Nave and Trapp, 2008).

Figure 2. The myelin sheath forms axonal domains which are essential for impulse conduction. (A) An electron micrograph from a longitudinal section from rat spinal dorsal root nerve showing the node of Ranvier flanked by internodal segments insulated by layers of compact myelin. Each layer of myelin terminates in a series of loops adjacent to the node of Ranvier (the paranodal loops) (B) Three axonal domains are defined by axon interactions with myelinating glia: the Na⁺ channel-enriched node of Ranvier, the adjacent paranode where the loops of myelin adhere to the axon through cell-adhesion molecules linked to the axonal cytoskeleton, the juxtaparanode which contains K⁺ channels and the internode sealed by compacted layers of myelin membrane to restrict transmembrane ion currents to the nodal region. Modified from Fields, RD, Trends Neuroscience, 2008

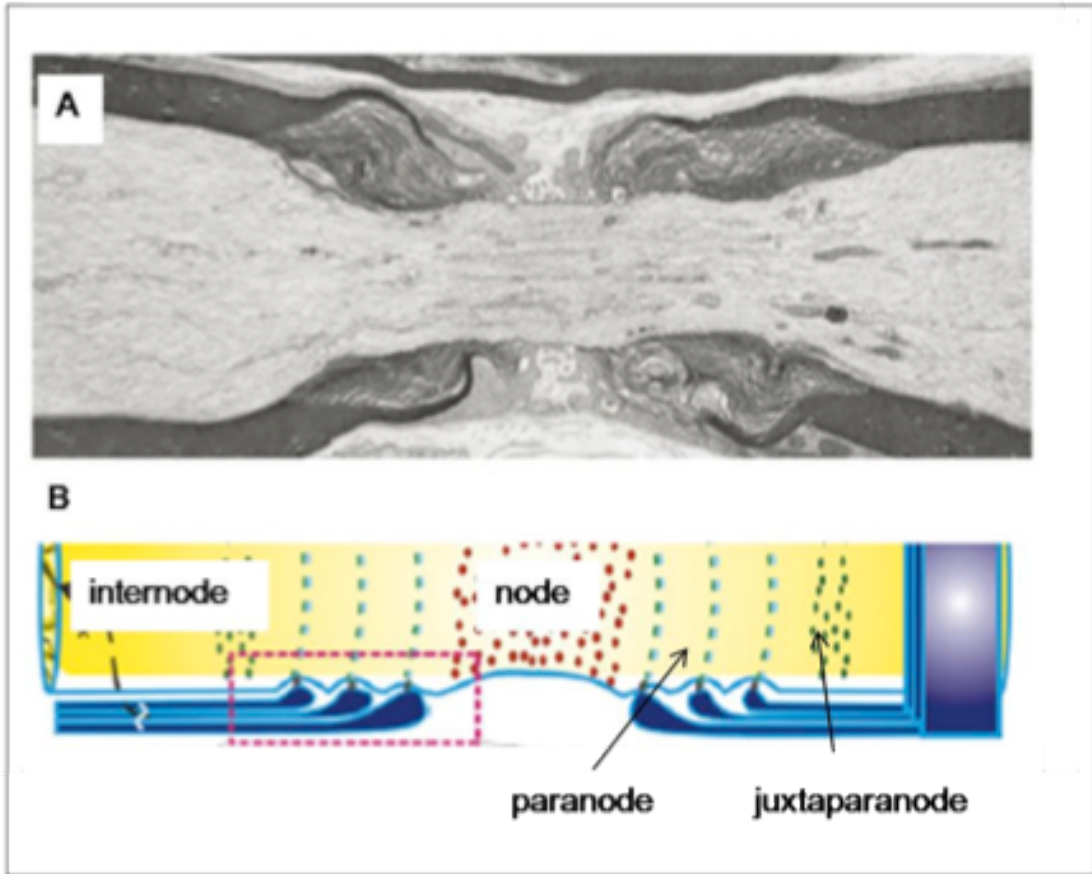
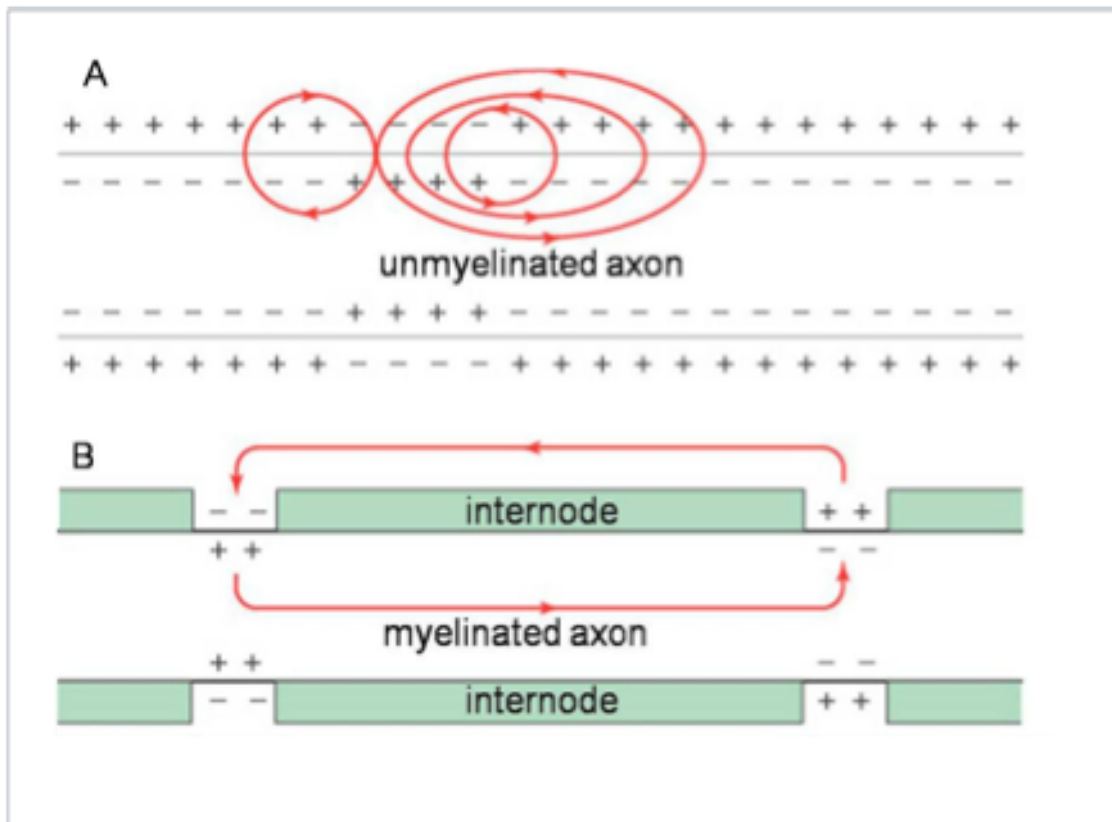


Figure 3. Comparison between conduction in a myelinated axon versus an unmyelinated axon. Arrows show the flow of action potential in local circuits into the active region of the membrane. (A) In unmyelinated fibers, the current flows throughout the length of the axon in contrast to (B) myelinated fibers where the current jumps between adjacent nodes resulting in saltatory conduction. Modified from Basic Neurochemistry, edited by Siegel, Albers, Brady and Price, Elsevier, 2006.



Myelin Composition

Myelin is composed of approximately 70% lipid and about 30 % protein, resulting in a highly hydrophobic membrane. This elevated degree of hydrophobicity and the compact multilamellar structure of myelin are responsible for its high insulating capacity.

Although no single lipid is unique to myelin or oligodendrocytes, the proportions of the various lipids are exclusive characteristics. When compared with other membranes and cells, both myelin and oligodendrocytes are highly enriched with glycosphingolipids (e.g. galactosylceramides) and their sulfated derivatives: sulfatides (e.g. sulfogalactosylceramides) which, combined, make up 20 percent of the lipids in myelin (Morell et al., 1973).

In contrast to the lipids, there are characteristic proteins unique to myelin and oligodendrocytes. Myelin basic protein (MBP) and proteolipid protein (PLP) represent about 80 percent of the total proteins present in myelin (Morell et al., 1973).

MBP exists in 3-4 major isoforms, depending on species, ranging in molecular weight from 14 to 21.5 kDa. Altogether, MBPs constitute about 30 percent of the myelin proteins (Boggs, 2006). The various MBPs are generated by alternative splicing of a single gene, allowing for precise biological control of protein isoform expression along development (de Ferra et al., 1985, Campagnoni, 1988). As further discussed in following chapters of this thesis, the differential localization of MBP isoforms in oligodendrocytes and myelin suggest

that some of these proteins may play a role in cell differentiation while others are mainly structural components of the myelin membrane (Pedraza et al., 1997). In this regard, studies in MBP-deficient mice have evidenced that MBP is an essential factor in myelin compaction (Readhead et al., 1987).

The other major myelin protein, PLP, exists in two isoforms (25 & 20 kDa), also originating from alternative splicing of a single gene (Nave et al., 1987). PLP may be acylated on cysteine residues, making it a hydrophobic proteolipid complex. Other myelin and oligodendroglial proteins include the enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) (Wolfgram, 1966), myelin-associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG). Antibodies raised against all of these proteins are frequently used as tools to study myelin formation and oligodendrocyte development.

Myelination: A Complex Developmental Process

The process of myelination in the developing brain is observed as a well-regulated event, conserved temporally and anatomically, yet the precise signals and regulatory mechanisms are still poorly understood. Oligodendrocyte migration and membrane synthesis must be tightly controlled to achieve proper myelination in the CNS. Given that oligodendrocytes only myelinate axons, it is evident that specific mechanisms of communication between neurons and oligodendrocytes must exist. It has been observed that electrical activity along the axon is essential for proper myelination, but specific molecular interactions between the axon and oligodendrocyte remain unclear (Tauber et al., 1980). The

expression of NMDA receptors in oligodendrocytes suggests that Ca^{2+} signaling in these glial cells is activated by axonal signals. Further support for signaling from neurons to myelinating glia is supported by previous findings from this laboratory indicating that oligodendrocytes respond to different neurotransmitters activating CREB (Sato-Bigbee et al., 1999), a transcription factor that controls proliferation, survival, and myelin protein expression in these cells (Afshari et al., 2001, Saini et al., 2004, Saini et al., 2005). In addition, the spatial coordination between neurons and myelinating oligodendrocytes may be subjected to control by astrocytes (Meyer-Franke et al., 1999).

Oligodendrocyte Development

As described previously, the formation of myelin during CNS development requires the integration of multiple signals and cells. In order to decipher the complex concert of events that result in proper myelination, it is critical to understand the development of oligodendrocytes. Mitotically active cells in the brain that are immunopositive for a chondroitin sulfate proteoglycan—NG2—are the progenitor cell population which gives rise to oligodendrocytes (Nishiyama et al., 1996). Different studies have indicated that the NG2^+ population may be multipotent with the ability to generate neurons and astrocytes, but 90 percent of its progeny is oligodendrocytes.

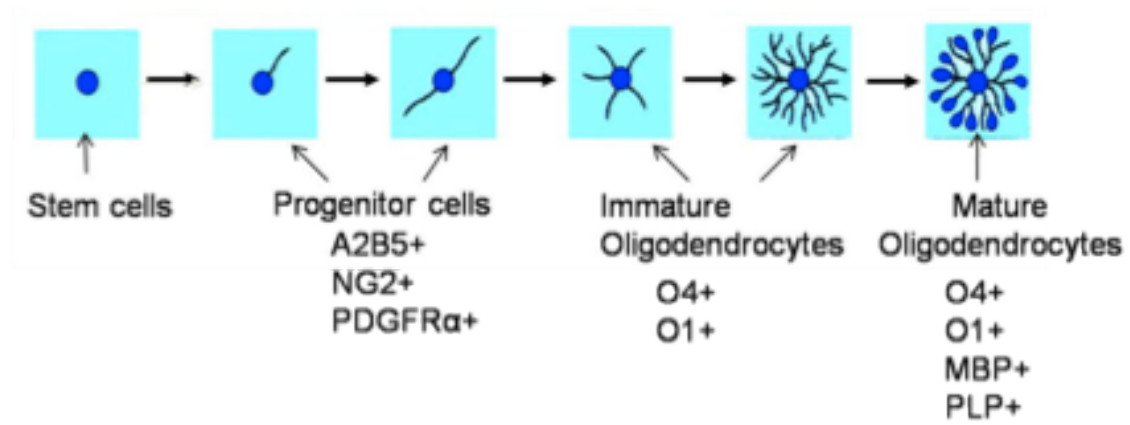
An oligodendrocyte progenitor cell will arise from an NG2^+ cell in ventricular zones of the brain and spinal cord (Luskin et al., 1988, Price and Thurlow, 1988). These oligodendrocyte progenitors are migratory and mitotically

active. They are characterized by the expression of certain transcription factors, including Olig1, Olig2, SOX10, and Nkx2.2 (Baumann and Pham-Dinh, 2001). Morphologically, oligodendrocyte progenitors appear as cells with a simple soma with one or two emerging polar processes.

Once the oligodendrocyte progenitor has migrated to the proper site, local growth factors drive its continuing maturation. Thyroid hormone (T_3) is a terminal differentiation signal to the oligodendrocyte progenitor, halting its mitotic activity and effectively inducing its conversion to a pre-oligodendrocyte (Almazan et al., 1985, Barres et al., 1994). These pre-oligodendrocytes can be identified by their expression of sulfatides, recognized by the O4 antibody (Bansal et al., 1989). The onset of CNPase expression is another hallmark of a developing oligodendrocyte. As they mature, pre-oligodendrocytes begin to form more complex networks of cell processes. Finally, the expression of MBP, PLP, MAG, and other characteristic myelin proteins will be observed when the cells develop into fully differentiated oligodendrocytes (Figure 4). This developmental sequence can be replicated *in vitro*, but proper myelin formation requires the presence of neurons and axon-glial interaction.

Such a dynamic process is under tight, yet fragile biological control. Many pathologies involving demyelination or dysmyelination represent an aberration in oligodendrocyte development. A greater understanding of the precise developmental controls will certainly reveal therapeutic targets for these diseases.

Figure 4. Stages in the development of oligodendrocytes. Each stage of oligodendrocyte differentiation is characterized by the expression of specific markers. Early progenitors are bipolar cells that can be labeled with the A2B5 antibody and express the PDGF- α receptor and the NG2 chondroitin sulfate proteoglycan. The next stage is represented by committed oligodendrocytes, multipolar cells that react with O4 antibody. The expression of the myelin proteins, including MBP and PLP, marks the final stage of differentiation.



Oligodendrocyte development must remain under precise biological control to ensure temporal and spatial fidelity. As indicated before, myelin has been shown to control both axonal radial growth and extension as well as to refine neuronal networks. Thus, understanding the factors controlling oligodendrocyte maturation and function could reveal therapeutic targets across the broad functionality of these cells.

The discovery of opioid receptors in oligodendrocytes raised questions about the potential effects of the endogenous opioid peptides on oligodendrocyte development. Work by Knapp et al. showed that oligodendrocytes express mu, delta, and kappa opioid receptors in a developmentally regulated manner. Moreover, oligodendrocytes also express their own endogenous opioids as levels of neuropeptide zymogens (proenkephalin and prodynorphin) were measured in those *in vitro* studies (Knapp et al., 2001).

Interestingly, activation of mu opioid receptor in oligodendrocyte progenitors resulted in increased proliferation. When the kappa opioid receptor was blocked, oligodendrocyte progenitors showed increased morphological differentiation whereas mature oligodendrocytes showed increased cell death.

Additional work has connected the kappa opioid receptor to pathology in the jimpy mouse, in which mutation in the myelin PLP gene causes oligodendrocyte death and severe CNS hypomyelination. These PLP deficient mice were shown to have a concomitant loss of the kappa opioid receptor in

oligodendrocytes. This 90% reduction is specific to the kappa opioid receptor and does not affect any other opioid receptors, suggesting that the kappa opioid receptor may be part of essential survival pathways. Taken together, these results suggest that endogenous opioids may serve as autocrine/ paracrine survival signals in oligodendrocyte development (Knapp et al., 2009). Control of opioid receptor levels as well as endogenous opioid expression may be essential to proper development and myelination.

In support of a crucial role of the opioid system in controlling myelin formation, recent *in vivo* studies from our laboratory have shown that perinatal exposure to buprenorphine affects myelination in the developing rat brain (Sanchez et al., 2008).

As described before, the mu partial agonist and kappa antagonist buprenorphine is currently used in clinical trials for pregnant opioid addicts. This drug appears to effectively prevent “street opioid” abuse by pregnant addicts as well as to decrease the incidence of neonatal abstinence syndrome. However, the effects of buprenorphine on child brain development remain unknown.

In our previous experiments, pregnant rats were implanted with minipumps to deliver buprenorphine at 0.3 or 1 mg/kg/day. While the lower dose is within the therapeutic levels of buprenorphine given to humans during gestation, 1 mg/kg/day correspond to a supra-therapeutic or abuse dose of the drug. Buprenorphine is known to cross into breast milk and thus, drug exposure via maternal milk was continued until the time of sacrifice or until weaning at 21

days postnatal. Analysis of the pup brains indicated that exposure to low, therapeutic doses of buprenorphine resulted in accelerated and increased expression of all MBP isoforms from postnatal day 12 to 26, corresponding to the normal period of rapid myelination in the developing brain. Conversely, perinatal exposure to the higher dose of buprenorphine resulted in delayed expression of all MBP isoforms, although levels recovered to normal values by postnatal day 26. Exposure to this elevated dose also resulted in a 25% decrease in the number of myelinated axons of the corpus callosum, a heavily myelinated structure that connects both cerebral hemispheres. These data are suggestive of a dose-specific mechanism controlling oligodendrocyte maturation and myelination in the developing brain.

Interestingly, analysis of the corpus callosum also indicated that, regardless of the dose, myelinated axons in pups exposed to buprenorphine exhibited increased axonal caliber accompanied by disproportionately thinner myelin sheath. Since the caliber of unmyelinated axons was not affected, this alteration of the normal myelin thickness/axonal diameter ratio suggests that disruption of endogenous opioid signaling by buprenorphine exposure alters oligodendroglial-neuronal interactions which are crucial to the coordination of myelin formation with radial axonal growth (Sanchez et al., 2008).

It is apparent that neuron-glia interaction is critical to proper myelination, but these data beg the question: where and how are opioids acting to control myelination in the developing brain?

The presence of opioid receptors on neurons and glial cells confounds any conclusions drawn from these data, so further studies are required to clarify any direct effects of buprenorphine on developing oligodendrocytes. To address this problem, experiments in this thesis investigated the potential direct effects of buprenorphine on cultured oligodendrocytes. The results indicated that buprenorphine indeed exerts direct dose-dependent effects that are highly specific for different stages of oligodendrocyte development. Moreover, similar effects were also observed for methadone, further stressing the need for detailed studies on the effects of opioid addiction treatments on brain development.

MATERIALS AND METHODS

Materials: Percoll, bovine pancreas DNase and papain for cell isolation as well as all cell culture medium components were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium/ Ham's F-12 (DMEM/F-12) (1:1) medium was obtained from Invitrogen (Grand Island, NY). Reduced growth factor Matrigel was from Becton Dickinson (Franklin Lakes, NJ). Buprenorphine, methadone, and the mu opioid receptor antagonist CTOP were purchased from Sigma-Aldrich (St. Louis, MO). All gel electrophoresis reagents and supplies were purchased from Bio-Rad Laboratories (Hercules, CA). The mouse anti- β -actin and rat anti-MBP (82-87 region) monoclonal antibodies were from Sigma-Aldrich and Millipore Corporation (Temecula, CA), respectively. The mouse O4 monoclonal antibody was kindly provided by Dr. Rashmi Bansal (University of Connecticut, Farmington, CT). Super Signal West Dura reagent was obtained from Pierce (Rockford, IL). Methyl-[^3H] thymidine (specific activity 75Ci/mmol) was from Amersham (Piscataway, NJ). All appropriate secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation and culture of oligodendrocytes: Timed-pregnant Sprague-Dawley rats were provided by Harlan Laboratories (Frederick, MD). Oligodendrocytes at different stages of development were directly isolated from 3- to 9-day-old rat brains as described previously (Colello and Sato-Bigbee, 2001) with minor modifications. For this, the brains were rapidly dissected out,

transferred to ice, and the meninges and main blood vessels removed by rolling the tissue on sterile filter paper. After mincing into 1-2 mm pieces, the tissue was incubated for 25 min. at 37°C in the presence of 1 unit/ml papain and 0.01 mg/ml DNase. Following incubation, the tissue was extensively washed and a total cell suspension prepared by forced filtration through a 75 µm pore size nylon mesh. The resulting suspension was centrifuged for 15 min at 30,000 x g in an isotonic self-generated Percoll gradient. The band enriched in oligodendrocytes was then subjected to differential adhesion on tissue culture-treated Petri dishes to eliminate microglial cells and residual astrocytes. The floating oligodendrocytes were then plated in 48-well plates (Falcon) (for western blot analysis) or on 10mm cover slips in 24-well plates (for immunocytochemistry) previously coated with 12.5 µL/well reduced growth factor-Matrigel extracellular matrix. Prior to use, the cells were maintained overnight in chemically defined medium (CDM) (DMEM) /F-12) supplemented with 1 mg/mL fatty acid-free bovine serum albumin, 50 µg/mL transferrin, 5 µg/mL insulin, 30 nM sodium selenite, 0.11 mg/mL sodium pyruvate, 10 nM biotin, 20 nM progesterone, 100 µM putrescine, 15 nM triiodothyronine. Astroglial contamination of these cultures, as assessed by glial fibrillary acid protein staining, was less than 5%. Animal use and isolation of oligodendrocytes were conducted in accordance with the guidelines from the National Institutes of Health and approved by the Virginia Commonwealth University Animal Care and Use Committee.

Western blot analysis: Pre-oligodendrocyte cultures containing equivalent numbers of cells per well were lysed in 80 μ L of 60 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate (SDS), and 5% 2-mercaptoethanol. 10 μ L samples were subjected to SDS-polyacrylamide gel electrophoresis in 15% acrylamide and the proteins were electrotransferred to nitrocellulose. The membranes were then subjected to immunoblot analysis as previously reported (Saini et al., 2005), with minor modifications. Nonspecific antibody binding to the blots was blocked by incubation in 10 mM Na_2HPO_4 , 2.7 mM KCl and 137 mM NaCl, pH 7.4, (PBS) containing 3% nonfat dry milk and 0.05% Tween-20 (blocking solution), for 1 hour at room temperature. Blots were then incubated overnight with anti-Myelin Basic Protein (dil. 1:100), an antibody that reacts with all 4 major MBP isoforms. β -Actin levels detected with anti- β -actin antibody (dil. 1:2,000) were used as loading controls. After extensive rinsing with PBS, blots were incubated for 30 min in blocking solution, followed by incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 2 hours. All antibodies were diluted in blocking buffer. After two 5-min rinses in PBS containing 0.05% Tween-20 and four 10-min rinses in PBS, the immunoreactive bands were detected by chemiluminescence with Super Signal West Dura reagent. The relative amount of immunoreactive protein in each band was determined by scanning densitometric analysis of the X-ray films using the NIH Image J program. After quantification of the bands, values were divided by β -actin levels to correct for loading differences.

[³H]Thymidine incorporation: After isolation, oligodendrocyte progenitors isolated from 3-day-old rat brain were plated on 48- well plates previously coated with 12.5 µL/well reduced growth factor Matrigel and maintained overnight in CDM. The next day, the medium was replaced with CDM containing 3 µCi/mL [³H]thymidine, in the presence or absence of different concentrations of buprenorphine or methadone. After 24 hours, the cultures were washed three times with ice-cold PBS, followed by incubation with 20% trichloroacetic acid (TCA) for 30 minutes at 4°C. After three 15 minute washes with 10% TCA, the cells were solubilized by incubation with 70% formic acid at 37°C for 1 hour. Aliquots were then used to determine the radioactivity by liquid scintillation counting.

Immunocytochemistry: Pre-oligodendrocytes were plated in 24-well plates (Fisher) on 10mm cover slips coated with 12.5 µL/well reduced growth factor Matrigel and maintained overnight in CDM. The next day, the medium was replaced with CDM alone or supplemented with buprenorphine. The medium was replaced every 48 hours. After 4 days of incubation, the cells were fixed in 4% paraformaldehyde and immunocytochemistry was carried out as previously reported (Sato-Bigbee et al., 1999). Non-specific antibody binding was blocked by incubation of the cells for 1 hour in PBS containing 5% non-fat dry milk, 0.05% Tween-20, and 0.5% normal goat serum (blocking solution). The cells were then

incubated overnight with the O4 antibody (dil.1:3) and MBP antibody (dil. 1:10) in blocking solution. After several washes in PBS, the cells were incubated for 30 min in blocking solution and for 2 hours with Alexa 488-conjugated anti- mouse IgM (dil. 1:250) or Texas Red-conjugated anti-rat IgM (dil. 1:150). The cultures were analyzed using a Nikon Eclipse 800M fluorescence microscope.

Statistical Analysis: Statistical analysis was performed by one-way analysis of variance using the GraphPad Prism program. Differences between results were considered statistically significant when p values were <0.05 .

RESULTS

As described before, our previous studies indicated that perinatal exposure to buprenorphine causes dose-specific and developmentally dependent effects on brain myelination. Elevated levels of MBP expression were observed at all studied ages in the brains of pups exposed to therapeutic drug doses. In contrast, treatment with a supra-therapeutic dose was accompanied by delayed MBP expression and reduced number of myelinated axons (Sanchez et al., 2008).

Because MBPs are considered to be markers of mature oligodendrocytes, these findings suggested that while therapeutic levels of buprenorphine accelerate and promote cell differentiation, higher buprenorphine doses may delay oligodendrocyte maturation. It is also important to consider that opioid receptors are known to be ubiquitously expressed and thus, the observed phenotypes may not necessarily reflect a direct action of buprenorphine on the oligodendrocytes but could be mediated through a number of other cell types, including neurons and different glial populations. Therefore, these hypotheses raise two major questions:

1. *Does buprenorphine have any effects on oligodendrocyte development?*
2. *Could those potential effects be attributed to direct or indirect actions of the drug?*

To address these two questions, we decided to investigate the potential direct effects of buprenorphine on cultured oligodendrocytes. Selection of the cell culture system for these experiments required special considerations.

First of all, opioid receptors are expressed throughout oligodendrocyte development, but previous studies have shown that their relative abundance changes over time (Knapp et al., 2001). In addition, oligodendrocyte progenitors, which are still mitotic, may respond quite differently to opioids than more mature pre-oligodendrocytes, the predecessors of the postmitotic and terminally differentiated cells capable of myelination. Therefore, it was critical that both progenitors and pre-oligodendrocytes be studied. Secondly, most studies on oligodendrocyte maturation use progenitors isolated from rodent newborn brain which are then cultured for several days to obtain cells at different stages of development. However, there is evidence to indicate that some responses may be lost when cells of the oligodendroglial lineage are maintained for extensive periods of time in purified cultures. As an example, neonatal oligodendrocytes become unresponsive to neurotransmitters when cultured for several days in the absence of neurons, losing their capacity to maintain neurotransmitter-activated signaling pathways coupled to Ca^{2+} mobilization (He et al., 1996). This becomes particularly important when trying to understand the molecular mechanisms that drive the last stages of differentiation leading to the generation of mature myelinating oligodendrocytes.

For these reasons, the cultures used in these studies were prepared using cells directly isolated from rat brain at different postnatal ages. It is expected that

these cells are—at the time of plating—more representative of their *in vivo* responses than those which are induced to differentiate for extensive periods of time in culture.

Cells isolated from 3-day-old rat brain are immature oligodendrocyte progenitors that are either bipolar or possess several simple processes and can be labeled with the A2B5 antibody. On the other hand, cells obtained from 9-day-old animals are pre-oligodendrocytes that may already be multipolar and react with the O4 antibody (Sato-Bigbee et al., 1999). These later cells represent a crucial developmental stage that immediately precedes the generation of mature oligodendrocytes capable of myelination.

TREATMENT OF OLIGODENDROCYTE PROGENITORS WITH BUPRENORPHINE INDUCES A DOSE-SPECIFIC STIMULATION ON CELL PROLIFERATION

We found that direct exposure to buprenorphine alters the proliferative capacity of the oligodendrocyte progenitors. In these experiments, DNA synthesis was assessed by incubating the cell cultures for 24 hours in chemically defined medium (CDM) containing ^3H -thymidine in the presence or absence of different concentrations of buprenorphine. As shown in Figure 5, exposure to buprenorphine resulted in a dose-dependent stimulation of cell proliferation, with maximal values of ^3H -thymidine incorporation observed between 0.5 μM and 1.0 μM buprenorphine. Interestingly, treatment with a higher concentration of the drug (3.0 μM) did not yield a significant increase in oligodendrocyte proliferation (Figure 5). These effects seem to be mediated by the mu opioid receptor because a similar dose-specific stimulation of cell proliferation was observed when the cultures were treated with methadone, a mu-selective agonist (Figure 6).

Figure 5. Exposure of oligodendrocyte progenitors to buprenorphine results in a dose-specific increase in cell proliferation. Oligodendrocyte progenitors were isolated from 3-day old rat brain as described under “Materials and Methods”. The cells were incubated for 24 hours in the presence or absence of different doses of buprenorphine. Proliferation was evaluated by ^3H -thymidine incorporation. The results are expressed as percentage of the control values \pm SEM. * $p < 0.05$.

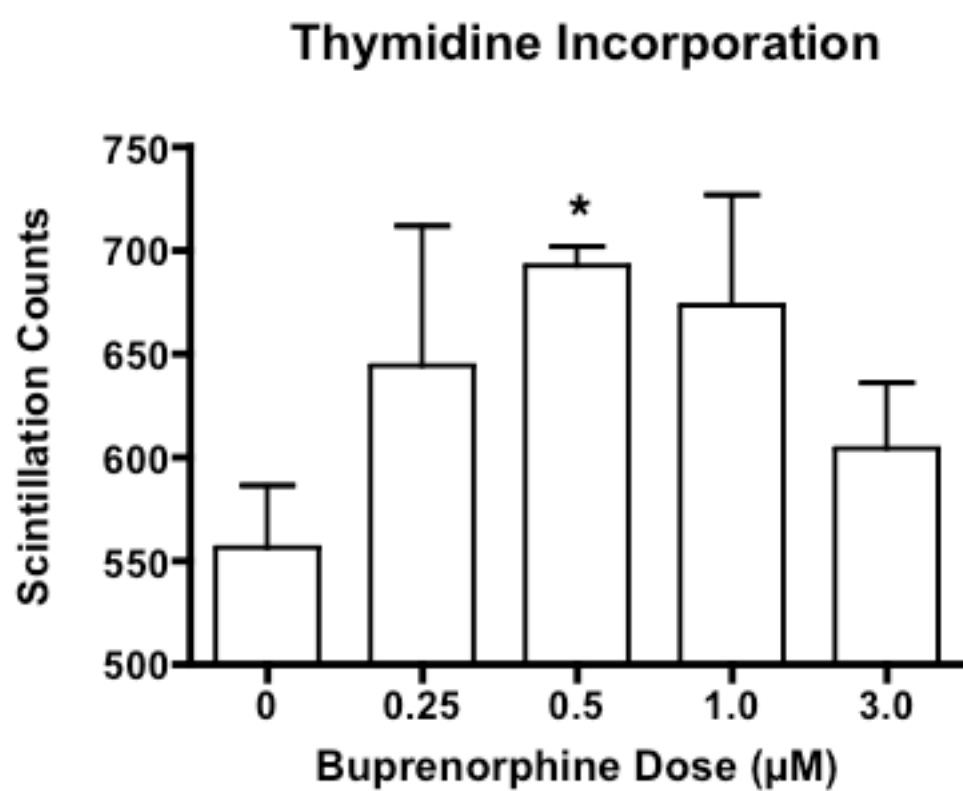
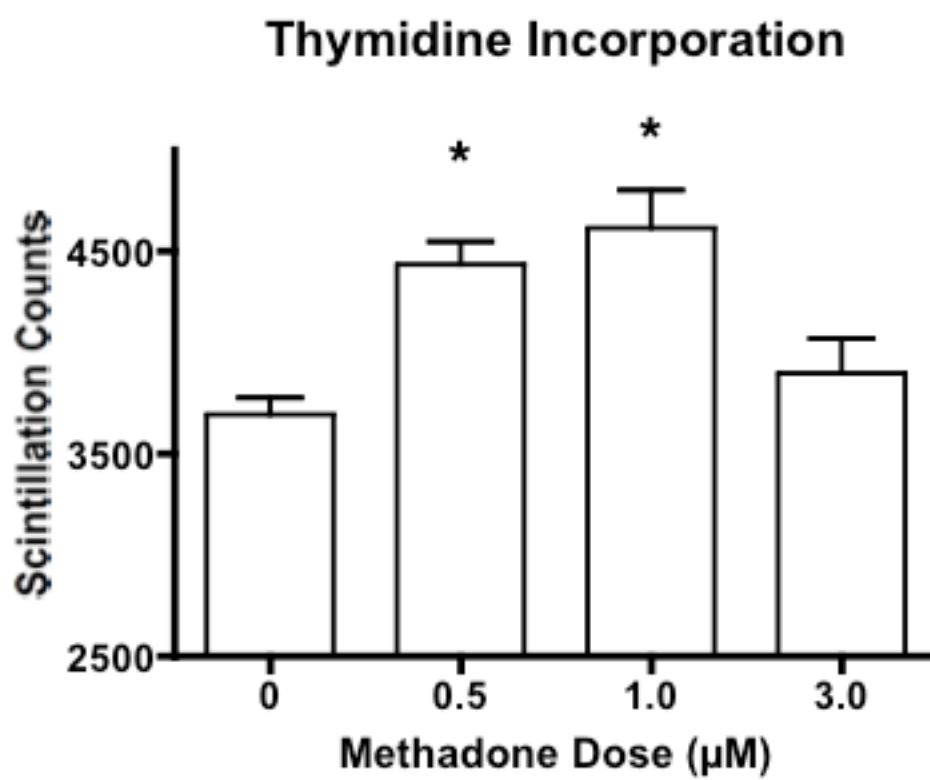


Figure 6. Exposure of oligodendrocyte progenitors to methadone also results in a dose-specific increase in cell proliferation. Oligodendrocyte progenitors were isolated from 3-day old rat brain as described under “Materials and Methods”. The cells were incubated for 24 hours in the presence or absence of different doses of methadone. Proliferation was evaluated by ^3H -thymidine incorporation. The results are expressed as percentage of the control values \pm SEM. * $p < 0.005$.



BUPRENORPHINE ALTERS PRE-OLIGODENDROCYTE DIFFERENTIATION AND MORPHOLOGY

In contrast with the observed effect on progenitor proliferation, buprenorphine affects the differentiation of pre-oligodendrocytes. Two approaches were used to assess differentiation: measuring MBP expression and morphological studies.

As seen in Figure 7, oligodendrocytes isolated from 9-day old rat brain showed a dose-specific response in MBP production when treated with buprenorphine for 4 days. A maximum stimulation was observed between 0.25 μ M and 0.5 μ M buprenorphine. Importantly, while these doses stimulated all four MBP isoforms, the 14 kDa isoform—predominant in mature myelin—showed the most significant increase. Interestingly, the maturity of the animals from which the cells were isolated was critical in achieving this stimulatory response. The increased MBP expression induced by low buprenorphine levels in cells from 9-day-old pups is not observed if the oligodendrocytes are instead isolated from 6-day-old animals (Figure 8), an observation that suggests that opioid signaling works in concerted action with other developmentally regulated mechanisms that control the last steps of oligodendrocyte maturation. Moreover, in cells from the younger animals, high buprenorphine doses reduced MBP levels to values below those corresponding to the controls, a finding which as discussed later, may implicate the participation of different opioid receptor types.

Moreover, in support of an effect of buprenorphine on cell differentiation, we also observe a dose-specific effect on the morphology of the cells (Figure 9). When treated with 0.5 μ M buprenorphine, which, as indicated above, induces maximum MBP stimulation, cells show significant increases in process outgrowth and membrane extension. Interestingly, this effect was abolished when cells were treated with higher buprenorphine doses (3.0 μ M), which also coincides with lower levels of MBP in western blotting analysis. Taken together, these observations indicate that buprenorphine exerts a direct effect on oligodendrocyte maturation.

Figure 7. Direct treatment of pre-oligodendrocytes with buprenorphine alters MBP expression in a dose-specific manner. Oligodendrocytes from 9-day-old rat brain were incubated for 4 days in CDM with or without 0.25, 0.5, 1.0, 3.0 μ M buprenorphine. MBP levels were determined by western blotting using beta-actin levels as loading controls. For correct quantification of individual MBP isoforms, film exposure times were adjusted to maintain linear detection of the bands. Figures correspond to representative experiments. Results in the bar graph are expressed as percentage of controls (0 μ M buprenorphine) \pm SEM. * $p < 0.02$.

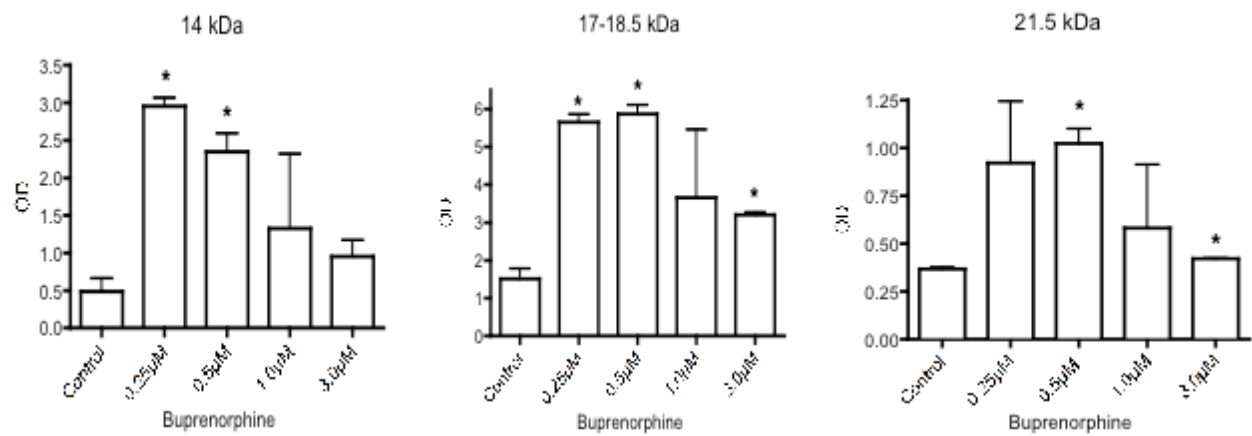
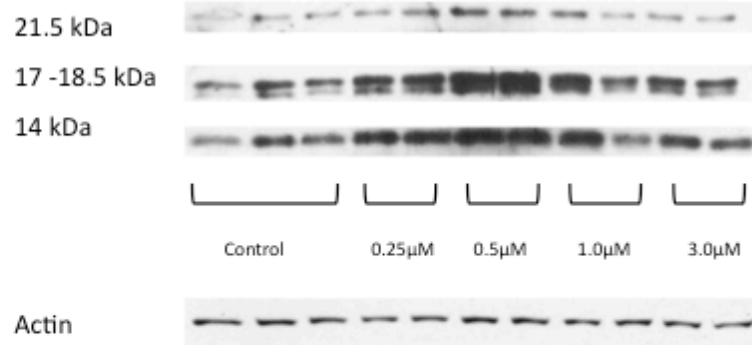


Figure 8. Direct treatment of oligodendrocytes from 6-day-old rat brain does not produce an increase in MBP levels. Oligodendrocytes were incubated for 4 days in CDM with or without 0.25, 0.5, 1.0, 3.0 μ M buprenorphine. MBP levels were determined by western blotting using beta-actin levels as loading controls. For correct quantification of individual MBP isoforms, film exposure times were adjusted to maintain linear detection of the bands. Figures correspond to representative experiments. Results in the bar graph are expressed as percentage of controls (0 μ M buprenorphine) \pm SEM. * p <0.05.

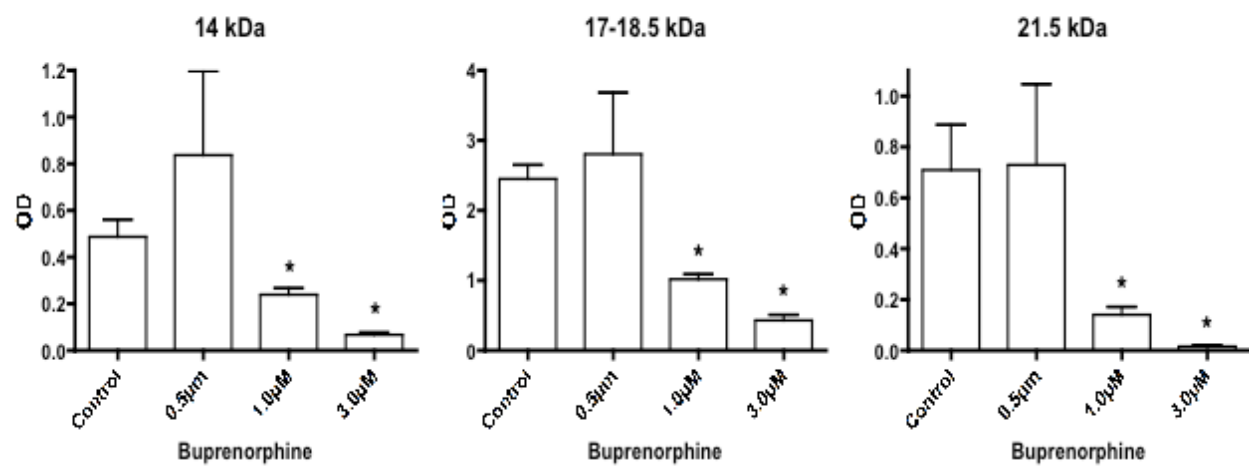
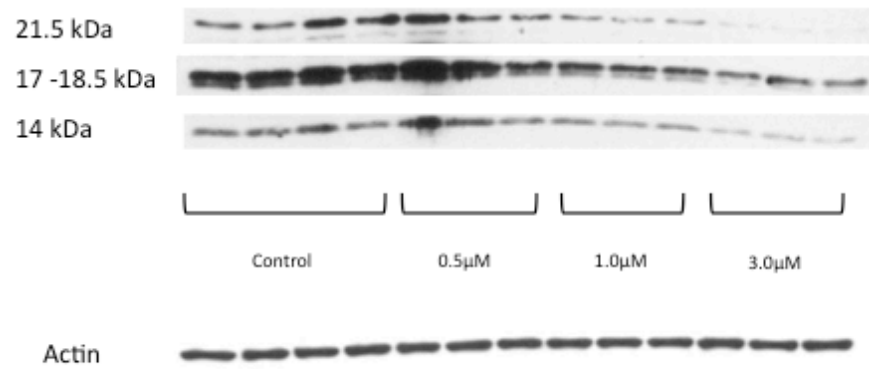
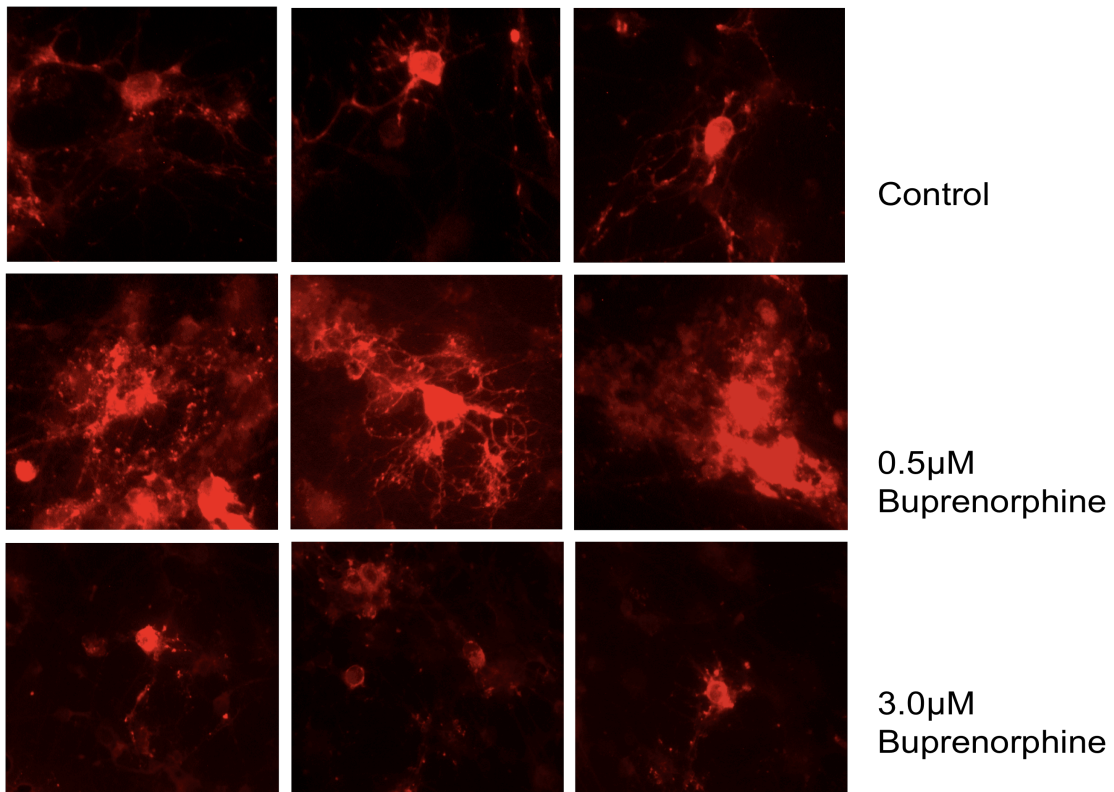


Figure 9. Buprenorphine treatment alters process network extension and membrane outgrowth in pre-oligodendrocytes in a dose-dependent manner. Oligodendrocytes isolated from 9-day old rat brain were cultured for 4 days in CDM with or without buprenorphine (0.5 μ M, 3.0 μ M). After fixation, cells were stained with anti-MBP antibody and visualized by fluorescent microscopy. The figure shows three representative panels corresponding to each of the treatment conditions. Note extensive process and membrane extension in cells treated with 0.5 μ M buprenorphine.



BUPRENORPHINE STIMULATORY EFFECTS ON CELL DIFFERENTIATION ARE MEDIATED BY THE MU OPIOID RECEPTOR

As indicated above, buprenorphine directly affects oligodendrocyte maturation. Because buprenorphine is both a mu partial agonist and a kappa antagonist, further investigation was focused on the specific receptor mediating these effects.

To address the role of the mu opioid receptor in this response we used two approaches. First, we investigated whether or not observed buprenorphine effects could be mimicked by methadone, a mu opioid receptor agonist. As seen in Figure 10, this is indeed the case, as methadone also induces dose-specific effects similar to those observed with buprenorphine treatment. Moreover, both methadone and buprenorphine effects are abolished by co-incubation with the highly specific mu opioid receptor antagonist CTOP (Figure 11) further supporting the role of this receptor in regulating oligodendrocyte maturation.

Figure 10. Direct treatment of pre-oligodendrocytes with methadone alters MBP expression in a dose-specific manner. Oligodendrocytes were incubated for 4 days in CDM with or without 0.25, 0.5, 1.0, 3.0, 5.0 μ M methadone. MBP levels were determined by western blotting using beta-actin levels as loading controls. For correct quantification of individual MBP isoforms, film exposure times were adjusted to maintain linear detection of the bands. Figures correspond to representative experiments. Results in the bar graph are expressed as percentage of controls (0 μ M buprenorphine) \pm SEM. * p <0.05.

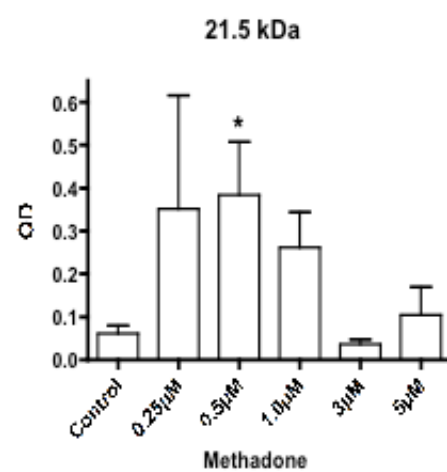
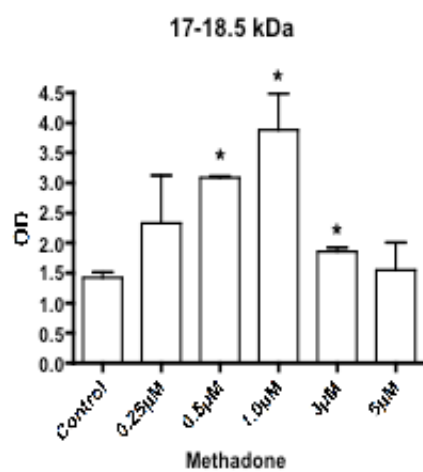
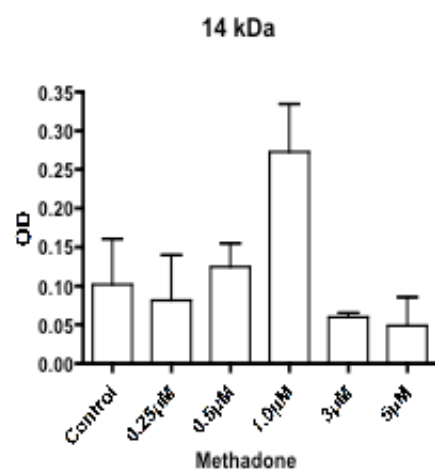
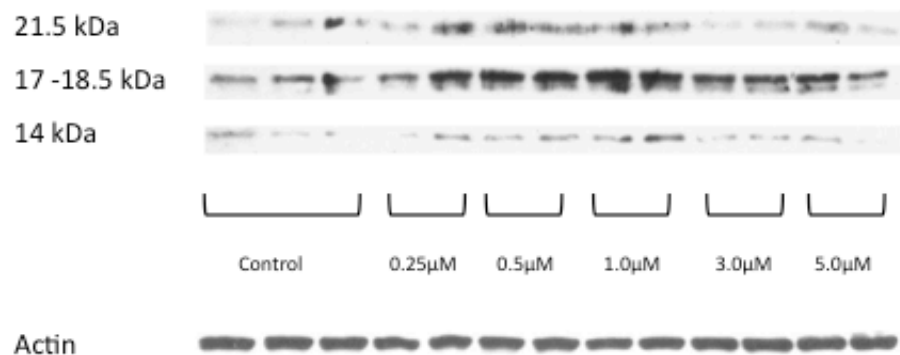
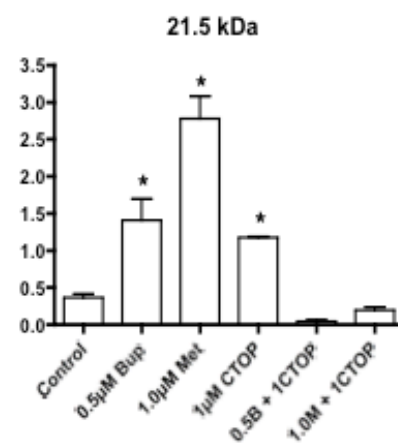
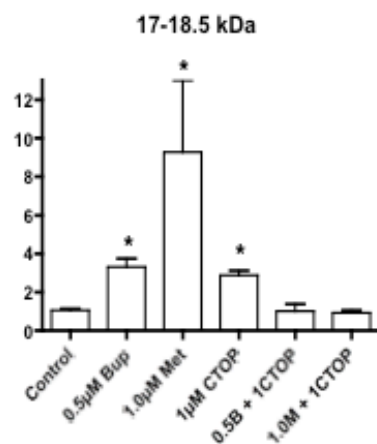
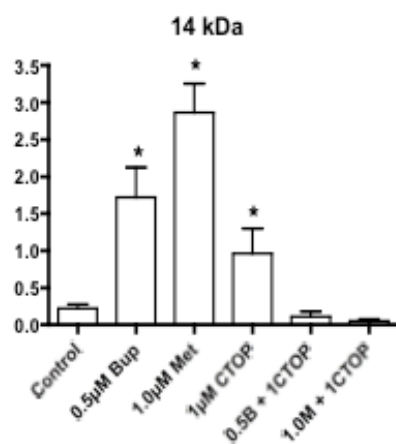
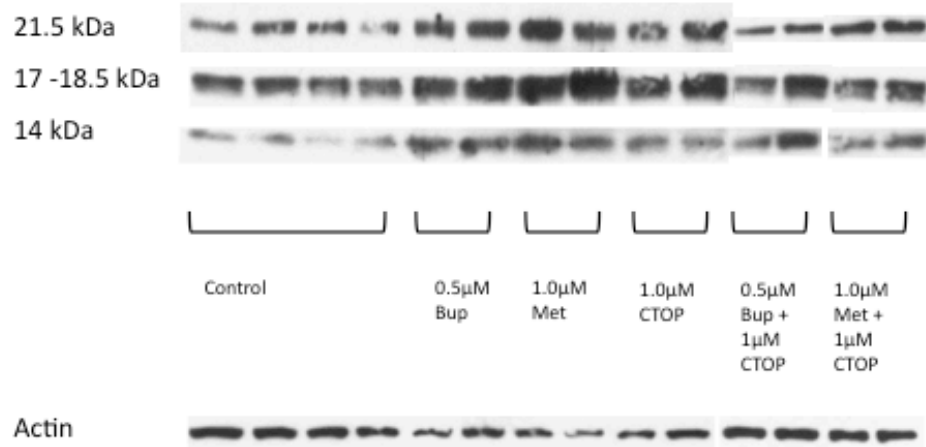


Figure 11. The mu opioid receptor antagonist CTOP blocks stimulation of MBP by buprenorphine and methadone. Oligodendrocytes isolated from 9-day old rat brain were cultured for 4 days in CDM in presence or absence of methadone (1 μ M) or buprenorphine (0.5 μ M) with and without CTOP. MBP levels were determined by western blotting using beta-actin levels as loading controls. For correct quantification of individual MBP isoforms, film exposure times were adjusted to maintain linear detection of the bands. Figures correspond to representative experiments. Results in the bar graph are expressed as percentage of controls (0 μ M buprenorphine).



DISCUSSION

Substitution therapies for opioid addiction, like those using methadone and buprenorphine, are valuable instruments in relieving the withdrawal associated with discontinuation of opioid abuse. At therapeutic doses, these synthetic opioids comprise the most effective tools available for substitution therapy. However, current understanding of their clinical efficacy is limited to the realm of adult opioid addicts.

Current clinical trials use buprenorphine to treat pregnant opioid addicts. Nevertheless, population studies have shown that children exposed to opioids *in utero* exhibit behavioral and cognitive deficits, suggesting that exposure to exogenous opioids may disrupt functions of the endogenous opioid system that could play a crucial role in the coordination of brain development.

As previously described, recent results from our laboratory showed that perinatal exposure to buprenorphine affects myelination in the developing rat brain (Sanchez et al., 2008). While exposure to therapeutic levels of buprenorphine resulted in accelerated and significantly increased brain levels of MBPs, supra-therapeutic levels retarded the appearance of these proteins and caused a decrease in the number of axons that were myelinated. Because MBPs are considered to be markers of mature oligodendrocytes, these findings suggested that while therapeutic levels of buprenorphine accelerate and promote cell differentiation, higher buprenorphine doses might delay oligodendrocyte maturation. However, opioid receptors are known to be ubiquitously expressed in

neuronal and glial populations of the CNS, making it difficult to parse out direct effects on any specific cell population. Thus, the reported effects of buprenorphine on myelination may not necessarily indicate a direct drug action on the oligodendrocytes. It is conceivable that buprenorphine effects on myelination may indirectly result from opioid actions on neurons and different glial populations which might then influence myelination. For this reason, it was important to investigate the possibility of direct effects of buprenorphine on the developing oligodendrocytes.

We have now found that (1) buprenorphine indeed exerts direct actions on the oligodendrocytes, (2) these effects are dose-specific, and (3) the direct actions of the drug are highly dependent on the stage of cell differentiation.

The present results showed that low buprenorphine doses induce increased proliferation of oligodendrocyte progenitors. In contrast, similar treatment of pre-oligodendrocytes show augmented capacity of the cells to synthesize MBPs and a remarkable increase in morphological complexity, both indicators of a stimulatory effect on oligodendrocyte maturation. These buprenorphine-dependent effects on cell proliferation and differentiation are primarily mediated by the mu opioid receptor.

Particularly interesting is the observation that oligodendrocyte progenitors, as well as pre-oligodendrocytes, exhibit a clear biphasic response to buprenorphine. Our data show an increase in oligodendrocyte progenitor proliferation following exposure to 0.5 μ M- 1.0 μ M buprenorphine. However, when

progenitors are treated with 3.0 μ M buprenorphine, rates of DNA synthesis do not significantly deviate from those of untreated cells. Likewise, pre-oligodendrocytes exhibit increased differentiation when exposed to low concentrations of buprenorphine but not in response to elevated doses of the drug. Morphine exposure was recently shown to increase apoptotic cell death of cultured oligodendrocytes (Hauser et al., 2009). Thus, it is possible to hypothesize that our present results may be due to an effect of buprenorphine on survival. However, preliminary experiments in which apoptosis was detected by TUNEL assay revealed no marked effects that could explain observed differences between controls and buprenorphine-treated oligodendrocytes.

Understanding of the molecular mechanisms underlying these effects is complicated by the complex pharmacology of buprenorphine. This drug is generally regarded as a partial mu opioid receptor agonist (Yu et al., 1997) and kappa opioid receptor antagonist (Leander, 1987). Additionally, buprenorphine is also known to bind to and activate the ORL-1 receptor (Bloms-Funke et al., 2000, Huang et al., 2001, Lutfy et al., 2003) and to exhibit both agonist and antagonist actions on delta opioid receptors (Sadée et al., 1982, Huang et al., 2001). Previous studies have shown that the mu opioid receptor is expressed very early in the oligodendroglial lineage, whereas the delta and kappa opioid receptors appear at later stages of development (Knapp et al., 1998). However, more recent analysis indicated the presence of the three opioid receptors at all stages along the oligodendroglial lineage (Hauser et al., 2009). Thus, it is difficult to ascribe responses to a particular opioid receptor signaling pathway. However,

our present results support the idea that the observed stimulatory actions of buprenorphine on the oligodendrocytes are mediated by agonist effect on the mu opioid receptor. This conclusion stems from the finding that these buprenorphine actions could be mimicked by replacing this drug with the mu agonist methadone. Furthermore, we also found that buprenorphine stimulatory effects were blocked when oligodendrocytes were co-incubated with CTOP, a selective and potent mu antagonist (Gulya et al., 1988, Hawkins et al., 1989, Law and Loh, 1999).

Previous studies showed that exposure of immature oligodendrocytes to the mu opioid receptor agonist PL017 resulted in elevated DNA synthesis (Knapp and Hauser, 1996), an observation that further supports the involvement of the mu opioid receptor as a mediator of the observed effects of buprenorphine on oligodendrocyte progenitor proliferation. Further supporting a role for opioids in regulating cell proliferation in the brain, treatment of adult neuroprogenitors with opioid antagonists results in a marked decrease in ERK 1/2 phosphorylation and levels of proteins involved in cell cycling (Persson et al., 2003). Although these adult progenitors may differ from their neonatal counterparts, we suspect that ERK remains a critical element in this effect of opioid signaling as this enzyme is known to play an important role in the induction of oligodendrocyte progenitor proliferation in response to growth factor stimulation (Cui and Almazan, 2007).

Importantly, the supposition of an *in vivo* role of the endogenous opioid system in controlling cell proliferation during CNS development is strengthened by the observation that synthesis of proopiomelanocortin and its processing into the mu and delta opioid receptor agonist beta-endorphin in the rat brain, are

elevated at embryonic days and postnatal ages that coincide with periods of crucial proliferative activity neuronal and glial progenitors (Angelogianni et al., 2000). Moreover, several lines of evidence support the idea that opioid signaling may not only be important during development but also play a role in maintaining adequate numbers of different cell populations in the adult brain. Treatment of cultured adult hippocampal progenitors with mu and delta opioid receptor antagonists decreases proliferation and neurogenesis (Persson et al., 2003). Additional work also shows that incubation with β -endorphin preferentially stimulates oligodendrogenesis in a population of cultured rat adult hippocampal progenitors (Raynor et al., 1994, Persson et al., 2006).

However, to our knowledge, the present study is the first to directly implicate the mu opioid receptor in the last stages of oligodendrocyte development. Direct exposure of pre-oligodendrocytes to buprenorphine not only stimulated MBP expression, a marker of cell maturation, but also resulted in a dramatic increase in process complexity and membrane outgrowth, raising the possibility that signaling through the mu opioid receptor plays a crucial role in controlling myelin formation. Moreover, this stimulation occurs in cells isolated from 9-day-old animals but not at earlier immediate time points of brain development. This later observation strongly suggests that this mu opioid receptor function is tightly coordinated with other developmentally controlled mechanisms that determine the last stage of oligodendrocyte maturation.

Thus, this work has shown that buprenorphine has direct effects on oligodendrocytes, and while these cells respond uniquely according to their stage

of development, we also found that dosage is critically responsible for the biphasic response that is curiously observed in both progenitors and pre-oligodendrocytes. Why do higher doses of buprenorphine fail to cause the increase in progenitor proliferation or stimulation of pre-oligodendrocyte differentiation that is observed when cells are treated with lower doses of this drug?

One potential explanation could be receptor desensitization. However, several lines of evidence suggest that a biphasic response could be due to simultaneous activation by buprenorphine of the ORL-1 receptor. Similar to the bell-shape curve observed in our experiments, others have shown that while lower doses of buprenorphine exert an analgesic effect, this antinociceptive action is significantly decreased by higher levels of the drug (Dum and Herz, 1981, Lizasoain et al., 1991). Later investigations also showed that while antinociception induced by buprenorphine is mediated by the mu opioid receptor, this effect is counteracted by the concomitant activation of ORL-1 receptors (Lutfy et al., 2003). In those studies, the co-administration of J-113397, an ORL-1 receptor antagonist, not only enhanced the antinociceptive effect of buprenorphine but also eliminated the characteristic bell-shape response. Moreover, the biphasic response was not observed in ORL-1 knockout mice. We have found that developing oligodendrocytes express ORL-1 (unpublished observations), although the potential role of this receptor in these cells has never been investigated before. However, it is tempting to hypothesize that while low doses of buprenorphine are able to activate the high affinity mu opioid receptor,

stimulating cell proliferation and differentiation, higher doses of the drug may counteract these effects by activation of ORL-1. It remains to be determined whether this type of dose-specific receptor activation may be responsible for the biphasic response of the oligodendrocyte cultures investigated in this thesis as well as our dose-dependent observations regarding MBP expression and myelination in our previous *in vivo* studies (Sanchez et al., 2008).

It is important to consider that the *in vivo* effects of buprenorphine on myelination may not be entirely explained by the direct effects of buprenorphine that we have now found on oligodendrocytes *in vitro*. There are several factors that confound any causative associations. First, as discussed above, dosage has been a critical element in each of the observed result. *In vivo* doses are measured in mg/kg/day without exact knowledge of the actual buprenorphine concentration in the CNS extracellular space, whereas cultures are maintained at a specific buprenorphine dose. In cultured oligodendrocytes, responses to buprenorphine vary based on the developmental state of the cells-- low doses of buprenorphine stimulate proliferation in progenitors, and differentiation in pre-oligodendrocytes. However, perinatal exposure to buprenorphine affects oligodendrocytes throughout their maturation. Responses to opioids vary temporally and because progenitor populations are maintained even in adult animals, effects of a specific dose on oligodendrocytes at a particular stage of development *in vitro* may not be completely predictive of *in vivo* effects.

In addition, *in vivo* phenotypes represent a concert of responses from neurons and glia. The ubiquity of opioid receptors in the CNS and the necessity

for axon-glia signaling in developmental coordination implicate all neurons and glia in the observed response. Since opioids influence neural progenitor differentiation and physiology of mature neurons and glia, their effects should be considered potent and diverse throughout the developing brain. Thus, while this work has focused on oligodendrocyte responses to opioids *in vitro*, our previous *in vivo* observations may also involve buprenorphine effects on neuron-glia interactions. Such interactions are critical to the proper timing of myelination—although difficult to prove, it is generally assumed that neurons signal to oligodendrocytes when to begin the process. In turn, oligodendrocytes control placement of protein complexes in the axonal membrane so that they will appose the nodes of Ranvier. Oligodendrocytes also control the axonal cytoskeleton and rates of vesicular transport (Simons and Trajkovic, 2006). When surface ligands and receptors bind, an array of signaling cascades are activated. Interestingly, some pathways have been shown to proceed through MAPK signaling which has previously been shown to mediate opioid effects in oligodendrocytes, suggesting a convergence of signaling in these developmental controls (Colognato et al., 2002, Persson et al., 2003, Colognato et al., 2004).

In support for an additional effect on neuron-glia interactions, we showed before that regardless of the dose, pups perinatally exposed to buprenorphine exhibited a significant increase in the caliber of the myelinated axons (Sanchez et al., 2008). Future studies should also focus on understanding whether inhibition of the kappa opioid receptor may also play role in the potential effects of buprenorphine on myelination. Surprisingly, these axons were characterized

by having a disproportionately thinner myelin sheath. These changes were also accompanied by increased levels of myelin associated glycoprotein (MAG), a molecule that has been implicated in glial-axonal communication (Yin et al., 1998). Exposure to therapeutic low buprenorphine doses also exhibited increased MAG glycosylation and interaction with the Src-family tyrosine kinase Fyn. Interestingly, others have implicated both MAG and Fyn as clue molecules potentially mediating bidirectional signals between neurons and oligodendrocytes (Biffiger et al., 2000). Based on these observations we hypothesized that opioid signaling may indeed be part of the molecular mechanisms that coordinate axonal radial growth with myelination (Sanchez et al., 2008).

Altogether, our previous and present findings suggest that interference with the endogenous opioid system during development may have significant consequences on oligodendrocyte maturation and myelination.

Although perinatal opioid exposure is the most immediate clinical correlate to this study, there are indeed other areas of clinical relevance. Many psychiatric disorders appear during adolescence and clinical studies of these patient populations show that changes in white matter volume coincide with onset of psychopathology. Under normal conditions, the rate of myelination increases during adolescence (Pfefferbaum et al., 1994). This is also a critical window for circuit refining and changes in the brain's reward system. The magnitude of these structural and functional changes is evident both physically and behaviorally (Paus et al., 2008). The onset of psychotic disorders during adolescence underscores the plasticity and vulnerability of the brain during this period.

Evidence suggests that many of these disorders are associated with abnormal development of myelin. For example, adolescents who engage in dangerous behavior have increased white matter maturity in the frontal cortex (Berns et al., 2009). Studies of white matter in psychotic patients show that pathology of myelin may contribute to the neurobiology of psychosis (Walterfang et al., 2005). Since we have shown the role of opioids in oligodendrocyte development and myelination, their role must be considered in these pathologies—both as a cause and as a possible therapy.

In conclusion, the results obtained in this thesis project indicate that buprenorphine and methadone exert direct and developmentally specific effects on the oligodendrocytes. Together with our previous observations, the present findings further support an important role for opioid signaling in regulating brain maturation and, in particular, the generation of oligodendroglial pools, oligodendrocyte maturation, and myelination. These observations stress the need for further studies and the strict control for the use of these drugs in the treatment of pregnant opioid addicts. Future studies investigating the molecular mechanisms by which buprenorphine and methadone affect myelination and neuro-glial interactions should provide deeper understanding into these developmental processes and new strategies for the managing of pregnant addicts.

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Publications

1. **Eschenroeder A**, Sanchez ES, Bigbee JW, Robinson SE, Sato-Bigbee, C. "Opioid addiction and brain myelination: buprenorphine and methadone alter oligodendrocyte development." Manuscript in preparation.
2. Chrestensen CA, **Eschenroeder A**, Ross, WG, Ueda T, Watanbe-Fukunaga R, Fukunaga R, Sturgill TW. "Loss of MNK function sensitizes fibroblasts to serum-withdrawal induced apoptosis." *Genes to Cells* (2007) 12, 1133–1140.
3. Chrestensen CA, Shuman JK, **Eschenroeder A**, Worthington M, Gram H, Sturgill TW. "MNK1 and MNK2 regulation in HER2-overexpressing breast cancer lines." *J Biol Chem*. 2007 Feb 16;282(7):4243-52.

Abstracts

1. Singh SK, Wilczynska KM, **Eschenroeder A**, Bryan L, Dumur K, Sato-Bigbee C, Kordula T. "Regulation and Role of YKL-40 in Glial Cell Function." 41st Annual Meeting of the American Society of Neurochemistry. March 6-10, 2010. Santa Fe, New Mexico.
2. **Eschenroeder AC**, Sanchez E, Bigbee JW, Robinson SE, Sato-Bigbee C. "Opioid Addiction and Brain Myelination: Direct Effects on Oligodendrocytes." 41st Annual Meeting of the American Society of Neurochemistry. March 6-10, 2010. Santa Fe, New Mexico.
3. Chrestensen CA, Shuman JK, **Eschenroeder A**, Worthington M, Gram H, Sturgill TW. "MNK1 and MNK2 Regulation in HER2 Over-expressing Breast Cancer Cell Lines." Targeting the Kinome. December 4-6, 2006. Basel, Switzerland.

Awards

1. Charles C. Clayton Award – for outstanding performance in the Department of Physiology and Biophysics Masters program